

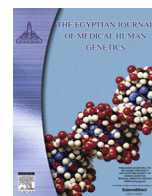
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Original article

Detecting *Mycoplasma pneumoniae* infections in nasopharyngeal specimens from Paediatric patients with asthma exacerbations in Baghdad: A Polymerase Chain Reaction – Gene based studyMuhi Kadhém Wannas Al-Janabi^a, Saad Hasan Mohammed Ali^{b,*}, Nadia Aziz Nasir^c, Muhammed Wehby^d^a Paediatrics Department, University of Baghdad, College of Medicine, Iraq^b Clinical Communicable Diseases Research Unit, University of Baghdad, College of Medicine, Iraq^c Community Medicine Department, University of Baghdad, College of Medicine, Iraq^d Ministry of Health, Children Welfare Teaching Hospital, Iraq

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ABSTRACT

Background: Numerous viral infections have triggered acute asthma exacerbations. Despite the fact that diagnosis of *M. pneumoniae* infection is based on sero-prevalence studies but molecular diagnostic techniques, such as PCR, have offered improvements in sensitivity, specificity and rapidity over the latest methods.**Objectives:** The aim of this molecular study is to determine the infection rates of *M. pneumoniae* in acute asthma exacerbation in a group of Iraqi children from Baghdad and also to examine the correlation of the disease with different variable characteristics and symptoms.**Methods:** This study included 94 children between 2 and 13 years old; Fifty in-patient asthmatic children and 44 non-asthmatic children as control group who were out-patients of the same hospital. Throat and nasal swab samples were taken for DNA extraction and PCR procedures.**Results:** PCR results show that 33.3% asthmatic patients were positive for *M. pneumoniae* while 66.7% were negative ($p < 0.001$). 53.8% of *M. pneumoniae*-positive asthmatic children were 2–5 years while 46.2% were 6–14 years old. Among asthmatic patients with positive PCR, 30.8% had positive history of seasonal pattern ($p = 0.026$) and 69.2% have positive family history of atopy ($p = 0.05$).**Conclusions:** Family history of atopy has strong association with asthma ($p = 0.005$), while factors such as sex, residence, seasonal allergen, animal allergen, passive smoking, mode of delivery or consanguinity has not been associated with asthma. *M. pneumoniae* in a respective bulk among pediatric patients with asthma constituted an important risk factor for asthma exacerbation presented as cough and wheezy chest without fever or chest X-ray findings.© 2017 Ain Shams University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Respiratory infections were reported to either cause wheezing episodes in children or to influence the onset as well as severity of asthma through both complex and intersecting mechanisms. Infections can also trigger atopic asthma while atopy in turn cause wheezing during airway infections and can modify the course of the disease [1].

Mycoplasma pneumoniae (*M. pneumoniae*) is an exceptionally small prokaryote, with no cell wall, insensitive to β -lactam antibiotics and Gram negative. It is a fastidiously growing bacterium, requiring the presence of a variety of substances such as nucleotides and sterols, for replication both in host and culture [2].

In humans, *M. pneumoniae* is among the most common organisms to cause acute respiratory infections in either children or adults [3]. It is also a major cause of community acquired pneumonia affecting 10–40% of children and young adults with clinical manifestations ranging from asymptomatic infection to fatal pneumonia or extra pulmonary diseases. However, the prevalence and association of *M. pneumoniae* in asthma is still not clear, especially in developing countries [4,5].

It is logical to believe, as others did, that infections with *M. pneumoniae* could play a greater role than it is readily considered

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for, regarding both the pathophysiology of asthma and acute exacerbations of bronchial asthma. In addition, we think that large numbers of infections with *M. pneumoniae* have been overlooked and not treated, and often it has been misdiagnosed as viral infections. Therefore, it seems important that this prospective molecular study made an attempt to assess the incidence and role of *M. pneumoniae* infection in children with acute exacerbation of bronchial and to make a correlation between bench and bedside such as making connection between the result of PCR for detecting *M. pneumoniae* DNA and the clinical and radiological findings.

2. Patients and methods

A prospective case-control study was performed on 94 children with age range of 2 to 13 years old. Fifty asthmatic children were admitted to the Children Welfare Teaching Hospital and 44 non-asthmatic children as outpatient visitors to the same hospital were included as age-matched control group. The study period was from July 1, 2013 to December 31, 2013.

This work has been carried out in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki) for experiments in humans.

For each child, a questionnaire form was filled asking the information about age, sex, growth parameter, number of wheezing attacks, response to bronchodilator, exposure to allergen, family history of asthma and atopy. Exclusion criteria of this study were those patients with failure to thrive as well as patients having associated diseases other than asthma. Each patient aged more than 6 years of age was sent for clinical tests such as chest X-ray, echocardiography, and pulmonary function tests. Throat and nasal swab samples were collected by taking stringent precaution and using sterile methods for collecting the samples. Samples were kept in special ready-to-use viral transport media (kept in collecting container filled with ice bags) and transferred immediately to the molecular laboratory of Clinical Communicable Diseases Research Unit/College of Medicine/University of Baghdad, for DNA extraction and PCR testing.

GENEKAM DNA ISOLATION KIT (Germany) was used to isolate DNA from the swabs containing the nasopharyngeal specimens, following manufacturer's instructions. Then *M. pneumoniae* amplification kit (manufactured by GENEKAM Biotechnology/Germany) was utilized to amplify the isolated DNA, following manufacturer's instructions. The cycling steps were as follows: initial denaturation at 95 °C for 9 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s, and a final extension of 7 min at 72 °C. PCR fragments were separated on 2% agarose gel, stained with ethidium bromide and visualized under UV light with trans-illuminator apparatus. Picture was taken with a gel documentation system.

Statistical analysis: Data were analysed using SPSS (Statistical Package for Social Science) program version 20. Chi-square was used as a test of significance for the qualitative data; P value ≤ 0.05 was considered to be statistically significant and a P value < 0.01 was considered highly significant.

3. Results

Thirty-nine asthmatic patients with a mean age of (6.33 ± 3.608) years old were included in this study along with forty-four non asthmatic patients as control group with mean age of 6.05 ± 3.444 years old. No statistical difference was found between means of age of cases and control (P value = 0.711). More than half (51.28%) of asthmatic children are 2–5 years old while the remaining 48.72% of asthmatic children were 6–14 years old.

The study shows that asthma is more common in males (27/39) than females (12/39) with male to female ratio were 2.2:1. No statistical significant difference as compared to control group as shown in Table 1.

The association of family history of allergen (including history of asthma, atopic dermatitis, and food allergy) in those asthmatic patients had showed statistically significance ($p \leq 0.05$) as compared to the control group while there was no statistical significance regarding the family history of allergic rhinitis in both groups as shown in Table 2.

PCR results show that *M. pneumoniae* was positive (Fig. 1) in 13/39 (33.3%) of asthmatic patients while negative in 26/39 (66.7%) asthmatic patients and all control group (44 out of 44; 100%) showed negative PCR results as shown in Table 3.

More than half (53.8%) of asthmatic children with *M. pneumoniae* had positive PCR results and were in the range of 2–5 years old age group, while 46.2% of them are in the 6–14 years old age group (Table 4). Among 13 asthmatic patients with positive PCR for *M. pneumoniae*, the male (9/13) to female (4/13) ratio was 2.2:1 ($p = 0.648$), where 7/13 were living in urban areas and the others have lived in rural areas ($p = 0.199$), 4/13 showed positive history of seasonal pattern ($p = 0.026$) and 9/13 showed positive family history of atopy ($p = 0.05$) (Table 5).

Table 6 shows the no of PCR-positive patients (i.e. having *M. pneumoniae* infections) and number of PCR-negative data in asthmatic patients according to their signs and symptoms of pneumonia where from 13 asthmatic patients with positive PCR for *M. pneumoniae*, 6/13 (46.15%) were presented with fever ($p = 0.135$), 10/13 (76.93%) have presented with cough ($p = 0.023$) and 11/13 (84.62%) have presented with wheezy chest ($p = 0.006$).

4. Discussion

4.1. Risk factors of asthma

Asthma as a chronic inflammatory disease of airways is characterized by hyper-responsiveness of airways to a multiple stimuli as well as a reversible airway limitation producing recurrent respiratory symptoms which are in the form of shortness of breath, cough and wheeze. The asthma pathogenesis seems to be the result of the influence of a complex mixture of several known factors, such as genetic, environmental, dietary change and occupation, recognized as predisposition factors to asthma [6,7].

Our study has found that family history of asthma is an important risk factor for this disease as showed up in 35.9% of asthmatic children in comparison to control group which showed only in 18.2% ($p = 0.05$). This result were consistent with other studies such as Burke in 2003 [8] Aws in 2005 [9], Nebal in 2007 [10] and Mahdi in 2010 [11].

Family history of atopic dermatitis also correlated as statistically significant ($p = 0.005$) and as a risk factor for childhood asthma where this result was detected in 41.1% as compared to 13.6% in the control group. This result is consistent with the results obtained by Aws in 2005 [9] and Ronchetti in 2001 [12] who found that a family history of atopy not only persisted but also strengthened over time. Our study did not found a significant correlation between childhood asthma and family history of allergic rhinitis ($p = 0.26$) which was 12.8% as compared to control group 20.5%, this result is consistent with the result reported by Nebal in 2007 [10].

It is generally agreed that atopy is an important risk factor for allergic diseases, such as asthma, rhinitis, and eczema, however the extent to which this atopy accounts for each of these diseases is controversial [13].

Table 1

Association between characteristics of asthma cases and controls.

Variable characteristics		Asthmatic n (%)	Controls n (%)	P value
Sex	Male	27(69.2%)	26(59%)	0.233
	Female	12(30.8%)	18 (41%)	
Residence	Rural	13(33.3%)	15(34%)	0.564
	Urban	26 (66.7%)	29(66%)	
Seasonal allergen	Negative	17(43.6%)	25(56.8%)	0.163
	Positive	22(56.4%)	19(43.2%)	
Animal allergen	Negative	24(61.5%)	31(70.5%)	0.266
	Positive	15(38.5%)	13(29.5%)	
Paternal smoking	Negative	14(35.9%)	15(34.1%)	0.523
	Positive	25(64.1%)	29(65.9%)	
Mode of delivery	NVD	25(64.1%)	30(68.2%)	0.436
	CS	14(35.9%)	14(31.8%)	
Consanguinity	Negative	19(48.7%)	27(61.4%)	0.175
	Positive	20(51.3%)	17(38.6%)	

n: number of patients.

Table 2

Association of family history of atopy between asthmatics and controls.

Family history		Asthmatic n (%)	Controls n (%)	P value
Family history of asthma	Positive	25(64.1%)	36(81.8%)	0.057
	Negative	14(35.9%)	8(18.2%)	
Family history of atopic dermatitis	Positive	23(58.9%)	38(86.4%)	0.005
	Negative	16(41.1%)	6(13.6%)	
Family history of food allergy	Positive	32(82.1%)	43(97.7%)	0.019
	Negative	7(17.9%)	1(2.3%)	
Family history of allergic rhinitis	Positive	34(87.2%)	35(79.5%)	0.265
	Negative	5(12.8%)	9(20.5%)	

n: number of patients.

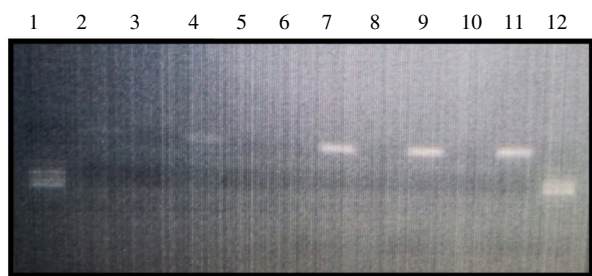


Fig. 1. Image of 2% Agarose gel electrophoresis for the detection of the PCR-amplicons of *M. pneumoniae* as seen under UV light via trans-illuminator apparatus where all positive samples showed bands between 309 and 339 bp.: Lanes 1 and 12 are 100 bp-DNA molecular markers (ladders); lanes 2, 4, 7 and 9 show positive PCR samples for *M. pneumoniae*; lanes 3, 5, 6 and 8 show negative PCR samples for *M. pneumoniae*; lane10 for negative control; and lane 11 for positive control.

These differences might be related to the type of study as it depends on parental accountability for the state of health of family members, which might result in an under-estimation of significance of some of these risk factors. However these results clearly

showed the importance of family atopy in the development of childhood asthma and supported the idea that asthma may be inherited to some extent.

This study showed no association between location of residence (whether rural or urban) and the development of asthma ($p = 0.56$), this result is consistent with study of Nebal in 2007 [10] and this may be due to the referee drainage area to our hospital.

Regarding the result of seasonal allergy and the passive smoking, both showed no associations between asthmatic group and control ($p = 0.16$ and $p = 0.52$, respectively). This controversy might be related to the accuracy of diagnosis of seasonal allergy and whether it is based on prospective follow up of patients or retrospective history from parents.

While in regards to passive smoking, it is logical to think that cigarette smoke, as irritant to the respiratory passages, is provoking asthma in a genetically predisposed child but in our study showed no association between asthmatic children and control group and this can be explained by the very high prevalence of active and passive smoking among different family members, but still the percentage of passive smoking among the asthmatic cases

Table 3Association of *M. pneumoniae* and asthma.

PCR	Asthmatics n (%)	Controls n (%)	P value
PCR results	Negative	26(66.7%)	44(100%)
	Positive	13(33.3%)	0(0%)
Total	39(100%)	44(100%)	

n: number of patients.

Table 4Difference between the mean of age of patients with positive and Negative PCR results for *M. pneumoniae*.

PCR		Number	Mean Age (years)	SD	P value
PCR Results	Negative	26	6.65	3.588	0.44
	Positive	13	5.69	3.706	

Table 5Association between characteristics of asthmatic children and *M. pneumoniae*.

Variable characteristics		PCR-negative n (%)	PCR-positive n (%)	P value
Sex	Male	18 (69.23%)	9 (69.23%)	0.648
	Female	8 (30.77%)	4 (30.77%)	
Residence	Rural	7 (26.92%)	6 (46.15%)	0.199
	Urban	19 (73.08%)	7 (53.85%)	
Seasonal allergen	Negative	8 (30.77%)	9 (69.23%)	0.026
	Positive	18 (69.23%)	4 (30.77%)	
Family history of allergy	Negative	16 (61.39%)	4 (30.77%)	0.05
	Positive	10 (38.61%)	9 (69.23%)	

n: number of patients.

Table 6Distribution of PCR results of *M. pneumonia* according to the signs and symptoms of pneumonia.

Sign and symptoms		PCR negative	PCR positive	P value
Fever	Negative	20 (76.29%)	7 (53.85%)	0.135
	Positive	6 (23.07%)	6 (46.15%)	
Cough	Negative	16 (61.54%)	3 (23.07%)	0.023
	Positive	10 (38.46%)	10 (76.93%)	
Wheezing	Negative	16 (61.54%)	2 (15.38%)	0.006
	Positive	10 (38.46%)	11 (84.62%)	
CXR	Normal	11 (42.3%)	4 (30.77%)	0.367
	Hyper inflated	15 (57.7%)	9 (69.23%)	

is still high, as shown in (64.1%) in case of positive history compared to (35.9%) with negative history.

There was also no significant association between animal allergen and asthma ($p = 0.26$), this result is consistent with the result reported by Medjo and co-workers in 2013 [14] who showed early, past and current pet-keeping were not significantly associated with asthma as well as, neither having cat nor a dog during childhood period was associated with asthma.

Regarding consanguinity, our study found no correlation with the development of asthma in childhood ($p = 0.17$) which in turn was consistent with the study of Aws in 2005 [9], although a study by Tarja and associates in 1998 [15] found that the presence of asthma in successive generations was more likely caused by shared genetic background than shared by environmental risk factors, and this is possible because of high level of consanguineous marriage in our community.

In regards to mode of delivery, our study found no association between childhood asthma and mode of delivery ($p = 0.43$). Similar results from Juhn et al. (2005) [16] and Maitra et al. in 2004 [17], who found that mode of delivery was not associated with a subsequent risk of developing childhood asthma or wheezing episodes. This may be due to the effect of mode of delivery on risk of developing asthma or wheezing episodes which varies over time. In addition, this result has been inconsistent because of potential selection as well as due to the ascertainment biases.

The present study does not show any association between the characteristics of asthma cases and controls group in regards to the use of paracetamol during pregnancy or early infancy ($p = 0.15$), this may be explained by the fact that paracetamol is an effective treatment for pain and fever in young children and should be a stock item in every parent's medicine cabinet.

In 2012, Bazaian [18] had found that intake of paracetamol during the first year of life is associated with lower respiratory symp-

toms, such as wheeze, cough and breathlessness, during the first three years of life. This association was seen even though the researchers accounted for the presence of lower respiratory tract infections, including pneumonia, bronchitis and bronchiolitis. Researchers have identified these types of infections as a potential confounder, as they are either more common in children with asthma or they are often treated with paracetamol. Even so, it is difficult to establish a cause and effect link in order to be able to categorically state that paracetamol usage during the first year of life has increased the risk of lower respiratory symptoms.

Although the researchers agreed on diagnosed respiratory symptoms, it is very difficult to exclude the possibility that the infants have been given more paracetamol due to presence of symptoms, even if an infection had not been diagnosed.

Importantly, there was no association between paracetamol intake and asthma when the children were aged seven. There was also no link between mothers' use of paracetamol and lung symptoms or asthma in their children.

Family history of food allergy shows significant associations with childhood asthma as our results show 17.9% in asthmatic patients compared to 2.3% in control group ($p = 0.019$). This result was consistent with study of Laura in 2010 [19], where children with food allergies were 3.8 times more likely to have asthma than those without food allergies. Food allergy has also appeared to be a marker for severity of asthma. Seven times more likely people with a food allergy get asthma attack.

4.2. *M. pneumonia* and asthma

The role of respiratory tract infections in the pathogenesis of asthma is well established where many viruses have an attribution in the exacerbation of asthma. Recently, the role of atypical pathogens such as *Chlamydia pneumoniae* and *M. pneumonia* in asthma

has become an active area of investigation [1]. *M. pneumoniae* has been primarily recognised as a causative agent of community-acquired pneumonia [1,4,5].

Recently *M. pneumoniae* infection has been linked to asthma in various ways, where an infection with this organism may precede the onset of asthma, exacerbate asthma, or play a part in asthma chronicity in some children and adults [20] which makes it more difficult to control [1].

The lack of either awareness or rapid and specific diagnostic techniques have made the growing importance of these pathogens either ill understood or undefined. Although the existing data are not conclusive, they are still suggestive enough to initiate the studies to evaluate their roles as possible mechanisms in the pathogenesis of asthma [1].

It is still not clear, the prevalence and association of *M. pneumoniae* with asthma, especially in the developing countries. To the best of our knowledge, this is the first kind of molecular study in Iraq. Initial studies have been conducted on *M. pneumoniae* by Esposito et al. in year 2000 [21] using PCR, and found that *M. pneumoniae* appears significantly more often in children with acute episodes of wheezing than in controls, where the infection was significantly associated with a history of recurrent wheezing. In 2009 in India, Avanish et al. [22] also reported the association of *M. pneumoniae* infection with acute exacerbations of asthma.

These results are consistent with our results which have demonstrated a statistically significant association between *M. pneumoniae* and asthma ($p \leq 0.001$), as the asthmatic group of children showed (33.3%) positivity for *M. pneumoniae* by PCR method as compared to none (0.0%) in the control group.

In contrast, the age, sex and location of residence in those asthmatic group with positive PCR result have showed no association when compared to those of negative *M. pneumoniae* ($p = 0.44$, $p = 0.64$ and $p = 0.19$, respectively). The real problem in interpreting these data is the techniques used to diagnose mycoplasma infections. For children nasopharyngeal aspiration or nasal lavage fluid samples have been used in PCR, and rates of positivity varies in children with asthma or pneumonia. This rate would probably be higher if the PCR was performed on broncho-alveolar lavage fluid samples, but it is difficult to routinely obtain such samples from children.

While in regards to seasonal allergy ($p = 0.02$) and family history of allergy ($p = 0.05$) both have showed significant correlations with *M. pneumoniae* in asthmatic patients, 69% for seasonal allergy and 38% for family history of allergy.

M. pneumoniae shows significant correlation with cough ($p = 0.02$) and wheezing chest ($p = 0.006$) as a presenting features on examination while has neither significant correlation with fever ($p = 0.13$) nor with chest X-ray findings ($p = 0.36$), this is mostly due to asymptomatic feature of this infection in the majority of cases.

5. Conclusions

Family history of atopy (asthma, atopic dermatitis, and food allergy) has showed strong association with asthma, while either

sex, location of residence, seasonal allergen, animal allergen, passive smoking, mode of delivery, usage of paracetamol or consanguinity showed no association with asthma. *M. pneumoniae* was highly associated with asthma and thus could be considered among the important risk factors for asthma exacerbation in our asthmatic pediatric patients, however, no important correlation between *M. pneumoniae* infection and age, sex and location of residence has been found. Cough and wheezy chest were considered important features of *M. pneumoniae*, while neither fever nor chest X-ray findings were correlated with this infection.

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